Mode of action of endogenous opiate peptides

As the culmination of a search for endogenous ligands of the opiate receptor[1–4], the structures of two closely related pentapeptides, isolated from porcine brain, with morphine-like properties in pharmacological tests, have been reported by Hughes *et al*[5]. The amino acid sequences of the pentapeptides, termed methionine enkephalin and leucine enkephalin, are Tyr-Gly-Gly-Phe-Met (Met^5-enkephalin) and Tyr-Gly-Gly-Phe-Leu (Leu^5-enkephalin), respectively. They behave as highly potent opiates in the mouse vas deferens and guinea pig ileum assays[5]. In addition, animals tolerant to morphine are also tolerant to enkephalin[6]. The action of the pentapeptides in the guinea pig ileum[5] and when injected intracerebrally or intracerebroventricularly[7–9] is very short lived, perhaps because of proteolytic degradation. A related peptide, with longer chain length has been shown to have a long duration of action[10]. Morphine and other narcotics have been shown to bind to the opiate receptor[11–13] and inhibit adenylate cyclase activity in homogenates of neuroblastoma×glioma hybrid cells[14,15], to inhibit cyclic AMP accumulation in intact hybrid cells[15,16] and in rat brain homogenates[17]. We have tested the effects of Met^5- and Leu^5-enkephalin on NG108-15 adenylate cyclase activity, and show here that endogenous opiate peptides are potent, receptor-mediated, inhibitors of adenylate cyclase of neuroblastoma×glioma hybrid cells.

NG108-15 hybrid cells were derived (unpublished results of B. Hamprecht, T. Amano and M.N.) by fusion of mouse neuroblastoma clone N18TG-2[18] with rat glioma clone

C6BU-1[19]. NG108-15 cells generate action potentials on electrical or chemical stimulation, synthesise acetylcholine (unpublished results of B. Hamprecht, T. Amano and M.N.), and form synapses with striated muscle cells[20]. They are richly endowed with opiate receptors[14].

The relationship between Met⁵-enkephalin, Leu⁵-enkephalin, and morphine concentrations and basal or prostaglandin E_1 (PGE_1)-stimulated NG108-15 adenylate cyclase activities are shown in Fig. 1*a* and *b*, respectively. The concentrations required for half-maximal inhibition of basal adenylate cyclase are 12, 40 and 1,500 nM, respectively, and 20, 120 and 1,500 nM for PGE_1-stimulated activity. Therefore, Met⁵-enkephalin and Leu⁵-enkephalin are approximately 100 and 25 times more potent than morphine as inhibitors of adenylate cyclase activity. The activities of Met⁵- and Leu⁵-enkephalin as inhibitors of adenylate cyclase are approximately equal to their activities as inhibitors of electrically evoked contractions of mouse vas deferens smooth muscle and are five times greater than their activities in a similar assay with guinea pig ileum[5].

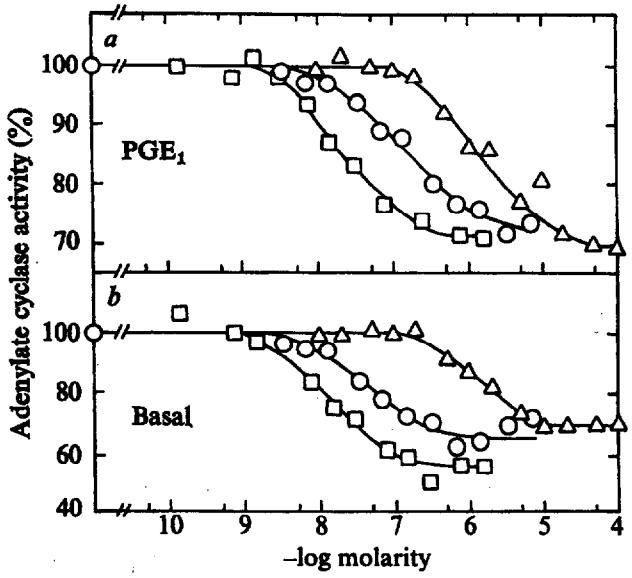


Fig. 1 Basal and PGE_1-stimulated adenylate cyclase activity of NG108-15 homogenates as a function of concentration of Met⁵-enkephalin (□), Leu⁵-enkephalin (○) or morphine (△). Reaction mixtures were incubated for 5 min at 37 °C with 97 μg of homogenate protein per tube. Other conditions are described in Table 1. Met⁵-enkephalin was synthesised by the rapid solid-phase procedure of Corley *et al.*[21]. The peptide product (approximately 80% pure) was purified by high-pressure liquid chromatography using a reversed phase system (Corasil, Waters Associates) and an acetonitrile, 0.1-M acetic acid gradient. Leu⁵-enkephalin, a synthetic product, was the gift of Drs R. Simantov and S. H. Snyder.

The effectiveness of Met⁵-enkephalin (1.6×10^{-7} M) as an inhibitor of adenylate cyclase decreases during incubation (Fig. 2), and is lost after approximately 20 min. The degree of inhibition by morphine (2×10^{-5} M) also decreases during incubation, but to a lesser extent. Further work is needed to determine whether Met⁵-enkephalin and morphine are inactivated during incubation or whether an activator of adenylate cyclase is formed.

The effect of naloxone, an opiate antagonist which competitively inhibits narcotic binding to the opiate receptor, on Met⁵-enkephalin or morphine-dependent inhibitions of adenylate cyclase are shown in Fig. 3*a* and *b*, respectively. Different concentrations of naloxone were used in the presence of 10^{-8}, 10^{-7} or 10^{-6} M Met⁵-enkephalin or in its absence. Naloxone completely reverses the inhibition of adenylate cyclase by each concentration of Met⁵-enkephalin tested. As expected for competitive interactions at a single site, the amount of naloxone required to reverse the actions of Met⁵-enkephalin or morphine is a function of the affinity of the receptor for the ligands and their concentrations. The dissociation constant of naloxone, K_e, calculated[22] from the data in Fig. 3*a* and *b* is 3×10^{-9} M for reversal of Met⁵-enkephalin inhibition and 2×10^{-9} for reversal of morphine inhibition (see legend to Fig. 3). These values agree well with the dissociation constant of naloxone of 2×10^{-9} M previously determined by direct measurement of the binding of ³H-naloxone to the opiate receptor of the hybrid cells[19].

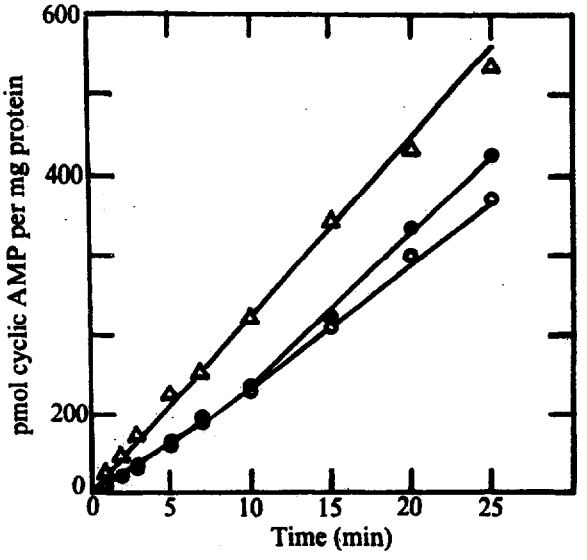
As Table 1 shows, Met⁵-enkephalin and morphine inhibit adenylate cyclase in homogenates prepared from NG108-15 hybrid cells, but not adenylate cyclase of the glioma parent, C6BU-1, which lacks narcotic receptors, or the neuroblastoma parent, N18TG2, which has fewer narcotic

Table 1 Effect of Met⁵-enkephalin and morphine on adenylate cyclase activity in homogenates of neuroblastoma × glioma hybrid and parental cell lines

Additions	Cell line: Hybrid NG108-15	Neuroblastoma N18TG-2	Glioma C6BU-1
	pmol cyclic AMP per min per mg protein		
None	41.4	13.2	15.8
Met⁵-enkephalin	27.2	14.7	15.1
Morphine	35.9	16.8	16.8
PGE_1	217	115	20.5
PGE_1 + Met⁵-enkephalin	168	120	18.4
PGE_1 + morphine	164	121	20.2

Adenylate cyclase activity was measured by the procedure of Salomon *et al.*[23] with modifications described before[15]. Each reaction mixture contained, in a final volume of 100 μl: 30 mM Tris-HCl, *p*H 7.5, 5 mM $MgCl_2$; 160 mM sucrose; 20 mM creatine phosphate; 10 U of creatine kinase; 1 mM ³H-cyclic AMP (10,000 c.p.m.); 0.5 mM RO20-1724 (0.1% ethanol final concentration); 1 mM α-³²P-ATP (10^6 c.p.m.); and where indicated, 0.16 μM Met⁵-enkephalin; 20 μM morphine sulphate; and 10 μM PGE_1. In addition, 90, 95 and 118 μg of homogenate protein was present for NG108-15, N18TG-2 and C6BU-1, respectively. Tubes were incubated for 3 min at 37 °C. Reactions were stopped with 1 ml of 5% trichloroacetic acid containing 1 mM ATP and 1 mM cyclic AMP. ³²P-cyclic AMP and ¹⁴C-cyclic AMP (for recovery) were counted after purification as described by Salomon *et al.*[23]. Cell lines, growth conditions and the adenylate cyclase assay method have been described previously (refs 14 and 15 and unpublished results of B. Hamprecht, T. Amans and M. N.). Washed cells, suspended in 0.32 M sucrose, 0.01 M Tris-HCl at *p*H 7.5 were stored at −80 °C before use.

Fig. 2 Time course of cyclic AMP formation by NG108-15 homogenates (97 μg per protein per tube) in the presence of 2×10^{-5} M morphine (○), 1.6×10^{-7} M Met⁵-enkephalin (●), or in the absence of added inhibitors (△). Assay conditions were as for Table 1.



receptors than NG108–15 hybrid cells[14,15]. These data, together with the naloxone reversal of enkephalin inhibition, show that both opiate receptors and enkephalin are required for the inhibition of adenylate cyclase activity.

The apparent inhibition constants for adenylate cyclase of Met⁵- and Leu⁵-enkephalins as well as morphine and some peptides which are fragments of enkephalin are summarised in Table 2. The K_i for Met⁵-enkephalin is 12–20 nM. Leucine enkephalin is three to six times less active an inhibitor than Met⁵-enkephalin and the enkephalin fragments tested had little or no effect on adenylate cyclase activity. In other experiments, not shown here, the di-and tripeptides did not antagonise morphine-dependent inhibition of adenylate cyclase. Met⁵- and Leu⁵-enkephalins are somewhat more potent inhibitors of basal, than PGE_1-stimulated adenylate cyclase. Morphine usually behaves similarly, although not in the experiment shown here. These observations show that opiate and PGE_1 receptors are functionally coupled either by allosteric interactions or by competition for the same site on the enzyme.

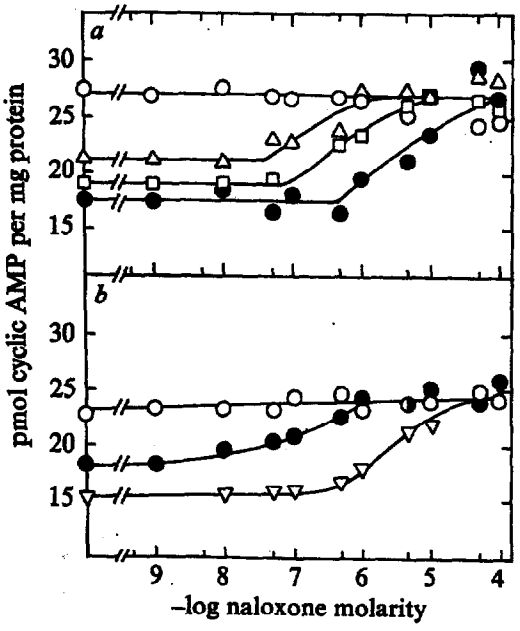


Fig. 3 *a*, relationship between naloxone concentration and the reversal of adenylate cyclase inhibition by 0.01 (△), 0.1 (□), or 1 μM (●) Met⁵-enkephalin. Also shown is the relationship between naloxone concentration and adenylate cyclase in the absence of Met⁵-enkephalin (○). Assays were for 5 min with 87 μg of homogenate protein per tube. *b*, a similar experiment without morphine (○), or with 1 μM morphine (●); or 100 μM morphine (▽). Reaction mixtures (90 μg of homogenate protein per tube) were incubated for 5 min. Other assay conditions are described in the legend of Table 1. Equilibrium binding constants (K_e) for naloxone were calculated by finding the concentration of naloxone at which the adenylate cyclase activity, in the presence of the highest concentration of inhibitor, is equal to that measured in the absence of naloxone at an inhibitor concentration 100 times lower. This concentration of naloxone is, by the dose-ratio method[28], equal to 99 times K_e.

Our experiments show that endogenous opiate peptides can be assayed rapidly by determining the opiate-peptide-dependent inhibition of adenylate cyclase which is reversed by naloxone. The range of the assay is 0.2–5.0 pmol of Met⁵ enkephalin per 100 μl of reaction mixture or 0.1–2.5 pmol per 50 μl of reaction mixture. Thus, we have found, in collaboration with Goldstein and Cox, that a peptide which has been purified extensively from pituitary tissue[24] and has a higher molecular weight than enkephalin is a potent inhibitor of both basal and PGE_1-stimulated adenylate cyclase of NG108-15 cells. Enkephalin antagonists can be assayed in a similar manner.

Table 2 Activity of peptides and morphine as inhibitors of adenylate cyclase in NG108-15 homogenates

Compound	K_i* (nM) Basal	PGE_1
Tyr–Gly–Gly–Phe–Met (Met⁵-enkephalin)	12	20
Tyr–Gly–Gly–Phe–Leu (Leu⁵-enkephalin)	40	120
Morphine	1,500	1,500
Tyr–Gly–Gly	>1,000,000†	—
Gly–Gly–Phe	>1,000,000‡	—
Phe–Leu	>1,000,000‡	—

Adenylate cyclase activity was determined as described in the legend of Table 1.

*Concentration of peptide required for half-maximal inhibition of basal or PGE_1 (10^{-6} M)-stimulated adenylate cyclase. Maximal inhibition of adenylate cyclase activity usually is 30–60% of total activity[15].

†Slight activity at 10^{-3} M (0–20% inhibition of adenylate cyclase).

‡No inhibition at 10^{-3} M.

An important question, that can be studied with cultures of NG108-15 cells, is whether the endogenous opiate peptides are addictive. As reported previously[25,26], NG108-15 cells and perhaps animals as well[27] chronically exposed to morphine, become tolerant to and dependent on morphine due to its dual action in inhibiting adenylate cyclase and eliciting a gradual increase in adenylate cyclase activity which compensates for the inhibition. Experiments are in progress to test whether hybrid cells become tolerant to and dependent on enkephalin and other opiate-like peptides.

Opiate peptides, 5–31 amino acid residues long[29,30], as well as β-melanocyte-stimulating hormone (β-MSH) apparently are derived by proteolysis of a common precursor, β-lipotropin[31], which had been isolated from pituitary[32]. Thus, β-lipotropin is a precursor of both an activator of adenylate cyclase (β-MSH)[33], and inhibitors of this enzyme (opiate peptides). Presumably, β-MSH and the endogenous opiates are recognised by different species of receptor which may be on the same or on different cells. Thus, positive and negative responses could result in separate cells or a single cell from different peptides derived from the same precursor.

The endogenous opiate peptides seem to be neurotransmitters or hormones which are destined for neurones with opiate receptors. The opiate peptide-receptor complex is a potent inhibitor of adenylate cyclase and thus the activations of adenylate cyclase by other species of neurotransmitters and hormones are suppressed. In effect, the opiate peptides act as pleiotropic desensitisers of many kinds of receptors which, in concert with the corresponding ligands, activate adenylate cyclase. Prolonged exposure to the opiate peptides may lead to an increase in adenylate cyclase activity as previously observed with morphine and other narcotics[25,26]. Thus, the opiate peptides may regulate the perception of incoming messages by neurones with opiate receptors in both a negative and positive manner.

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1 Hughes, J., *Brain Res.*, **88**, 295–308 (1975).
2 Terenius, L., and Wahlström, A., *Acta physiol. scand.*, **94**, 74–81 (1975).
3 Pasternak, G. W., Goodman, R., and Snyder, S. H., *Life Sci.*, **16**, 1765–1769 (1975).

4 Teschemacher, H., Opheim, K. E., Cox, B. M., and Goldstein, A., *Life Sci.*, 16, 1771–1776 (1975).
5 Hughes, J., *et al.*, *Nature*, 258, 577–579 (1975).
6 Waterfield, A. A., Hughes, J., and Kosterlitz, H. W., *Nature*, 260, 624–625 (1976).
7 Bolhuis, J. D., *et al.*, *Nature*, 260, 625–626 (1976).
8 Fert, A., Simantov, R., and Snyder, S. H., *Proc. natn. Acad. Sci. U.S.A.* (in the press).
9 Chang, J. K., Fong, B., Fert, A., and Fert, C. B., *Life Sci.* (in the press).
10 Feldberg, W. S., and Smyth, D. G., *J. Physiol., Lond.* (in the press).
11 Fert, C. B., and Snyder, S. H., *Science*, 179, 1011–1014 (1973).
12 Terenius, L., *Acta Pharmac. Tox.*, 32, 317–320 (1973).
13 Simon, E. J., Miller, J. M., and Edelman, I., *Proc. natn. Acad. Sci. U.S.A.*, 70, 1947–1949 (1973).
14 Kiss, W. A., and Nirenberg, M., *Proc. natn. Acad. Sci. U.S.A.*, 71, 3474–3477 (1974).
15 Sharma, S. K., Nirenberg, M., and Kiss, W. A., *Proc. natn. Acad. Sci. U.S.A.*, 72, 390–394 (1975).
16 Traber, J., Fischer, K., Larzin, S., and Hamprecht, B., *Nature*, 253, 120–122 (1975).
17 Collier, H. O. J., and Roy, A. C., *Nature*, 248, 24–27 (1974); *Prostaglandins*, 7, 361–376 (1974).
18 Minna, J., Glazer, D., and Nirenberg, M., *Nature new Biol.*, 235, 225–231 (1972).
19 Amano, T., Hamprecht, B., and Kemper, W., *Expl Cell Res.*, 85, 399–408 (1974).
20 Nelson, P., Christian, C., and Nirenberg, M., *Proc. natn. Acad. Sci. U.S.A.*, 73, 123–127 (1976).
21 Corley, L., Sachs, D. H., and Anfinsen, C. B., *Biochem. biophys. Res. Commun.*, 47, 1353–1359 (1972).
22 Kosterlitz, H. W., and Watt, A. J., *Br. J. Pharmac.*, 33, 266–276 (1968).
23 Salomon, Y., London, C., and Rodbell, M., *Analyt. Biochem.*, 58, 541–548 (1974).
24 Cox, B. M., Opheim, K. E., Teschemacher, H., and Goldstein, A., *Life Sci.*, 16, 1777–1782 (1975).
25 Sharma, S. K., Kiss, W. A., and Nirenberg, M., *Proc. natn. Acad. Sci. U.S.A.*, 72, 3092–3096 (1975).
26 Traber, J., Gullis, R., and Hamprecht, B., *Life Sci.*, 16, 1863–1868 (1975).
27 Collier, H. O. J., Francis, D. L., McDonald-Gibson, W. J., Roy, A. C., and Saeed, S. A., *Life Sci.*, 17, 85–90 (1975).
28 Guillemin, R., Ling, N., Burgus, R., *C.r. hebd. Séanc. Acad. Sci.*, Series D., 282, 783–785 (1976).
29 Li, C. H., and Chung, D., *Proc. natn. Acad. Sci. U.S.A.*, 73, 1145–1148 (1976).
30 Bradbury, A. F., Smyth, D. G., and Snell, C. R., *Nature*, 260, 793–795 (1976).
31 Bradbury, A. F., Smyth, D. G., and Snell, C. R., in *Peptides: Chemistry, Structure and Biology* (edit. by Walter, R., and Meienhofer, J.), 609–615 (Ann Arbor Science, Inc., 1975).
32 Li, C. H., *Nature*, 201, 924 (1964).
33 Braun, T., Hechter, O., and Li, C. H., *The Endocrine Society*, Fifty Second Meeting, 48 (1970).